EXHIBIT C

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Neuromedin B Is Present in Lung Cancer Cell Lines[1]

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Abstract

Previously, high levels of gastrin-releasing peptide and its mRNA were detected in classic small cell lung cancer cell lines. Here the ability of lung cancer cell lines to synthesize neuromedin B (NMB), a structurally similar mammalian bombesin-like peptide, was investigated. By radioimmunoassay, NMB (0.1–0.7 pmol/mg of protein) was detected in 23 of 33 lung cancer cell lines. In contrast, gastrin-releasing peptide (0.1–12.9 pmol/mg of protein) was detected in 16 of 32 cell lines. Using gel filtration and high pressure liquid chromatography techniques, the main peak of immunoreactive NMB coeluted with synthetic NMB. By Northern analysis, a 0.8-kilobase mRNA species was present, using poly(A) mRNA derived from two of three lung cancer cell lines. Using a more sensitive S1 nuclease protection assay, NMB mRNA was present in most of the 15 lung cancer cell lines examined. These data suggest that NMB may be a regulatory peptide in lung cancer.

Introduction

NMB[3] and GRP are two known mammalian members of the BN family of peptides. NMB is a 10-amino acid peptide which has seven of the same 10 carboxyl-terminal amino acids as does GRP (1). A cDNA clone for human prepro-NMB was isolated from a human hypothalamic library and localized to chromosome 15 (2). NMB is derived from a 116-amino acid protein (2) which contains a 24-amino acid signal sequence at the amino-terminal, followed by a 32-amino acid NMB-like peptide and a carboxyl-terminal extension peptide. NMB-32 may then be metabolized by a trypsin-like enzyme to yield NMB (3).

While the role of NMB in lung cancer remains unknown, GRP functions as an autocrine growth factor in some SCLC cells (4). The gene for GRP was cloned from a human lung carcinoid biopsy specimen and SCLC cell lines and is localized to chromosome 18q21 (5, 6). Type I prepro-GRP contains a 23-amino acid signal sequence followed by a 27-amino acid GRP, a glycine amidation donor, a dibasic cleavage site, and a 95 amino acid carboxyl-terminal extension peptide. GRP may be metabolized by trypsin-like enzymes to GRP^{18-27}, which is present in and secreted from classic SCLC cell line NCI-H345 (7, 8). GRP binds with high affinity to cell surface receptors (9), causes phosphatidylinositol turnover (10), elevates cytosolic Ca^{2+} (11), and stimulates the growth of SCLC *in vitro* (12) and *in vivo* (13). The autocrine growth cycle of SCLC may be disrupted by BN receptor antagonists (14).

Here the expression of NMB in lung cancer cell lines was investigated. NMB was detected by radioimmunoassay in most lung cancer cells. Also, NMB mRNA was present in most lung cancer cell lines examined.

Materials and Methods

Cell Culture. Human lung cancer cell lines were cultured in serum-supplemented medium (RPMI 1640 containing 10% heat-inactivated fetal bovine serum) at 37°C, as described previously (15,16). The NSCLC cell lines were split weekly 1:20, using trypsin/EDTA. The SCLC cell lines grew as floating aggregates and were split 1:1 weekly. Routinely, the cells had >90% viability, were *Mycoplasma* free, and were used when they were in exponential growth phase.

Radioimmunoassay. For RIA, the cells were rinsed 3 times in phosphate-buffered saline, the peptides were extracted in boiling 2 N acetic acid, and the samples were sonicated and centrifuged at 10,000 × *g* for 10 min. The supernatants were frozen and lyophilized. The supernatants were resuspended in RIA buffer phosphate-buffered saline containing 0.25% bovine serum albumin and assayed for immunoreactive NMB (17). ^{125}I-Bolton-Hunter-NMB (5000 cpm) was incubated with heterologous antiserum (1/50,000) in the presence or absence of competitor (the total volume was 400 μl); the limit of sensitivity of the RIA was 10 fmol and the linear range was 10–50 fmol. After 16 h at 4°C, 100 μl of normal rabbit serum (1/100), 100 μl of goat anti-rabbit serum (1/10), and 200 μl of 12% polyethylene glycol were added. After 30 min at room temperature, the tubes were centrifuged at 1000 × *g* for 25 min, the supernatants were removed, and the pellets were assayed for radioactivity. For gel filtration studies, the lyophilized extracts were resuspended in 0.1 N acetic acid and fractionated on a 50- x 1.5-cm Pharmacia G-25 superfine column. The column was eluted with 0.1 N acetic acid, and 1-ml fractions were collected. The fractions were frozen, lyophilized, and assayed for immunoreactive NMB. For HPLC studies, the tissue extracts were partially purified using a C18 Sep-Pak column, as described previously (18). The samples were then applied to a μBondapak/C18 column and eluted with an 18–33% acetonitrile gradient in triethylammonium phosphate (0.25 M, pH 3). One-ml fractions were collected for 60 min, aliquots were removed, and the fractions lyophilized and assayed for immunoreactive NMB. Protein was determined using the method of Lowry *et al.* (19).

mRNA. The cells were lysed in 4 M guanidine isothiocyanate and layered on top of a 5.7 M cesium chloride gradient (20). Total RNA was pelleted by centrifugation at 100,000 × *g* for 20 h. The pellet was resuspended in 0.3 M sodium acetate, extracted with SS-phenol/chloroform, washed with chloroform, and precipitated with ethanol. Total RNA was resuspended in water and stored at −70°C. The 260/280 absorbance ratio was 1.5–2.0. RNA (500 μg) was isolated from lung cancer cell lines and poly(A) RNA was purified from the total RNA using oligo(dT)-cellulose chromatography (21). Ten μg of denatured poly(A) RNA were placed in loading buffer and separated in a 0.66 M formaldehyde-1% agarose gel for 2–3 h at 200 V (22). Formaldehyde was removed by incubating the gel in 10× SSC [3 M NaCl 0.3 M Na citrate (pH 7.0)] for 20 min. The gel was blotted on nitrocellulose membrane overnight. The membrane was baked for 2 h at 80°C and incubated at 42°C with hybridization buffer (10% dextran sulfate, 40% formamide, 4× SSC, 0.2 M Tris, 1× Denhardt's solution, 20 μg/ml salmon sperm DNA) containing 0.5 × 10^6 cpm/ml ^{32}P-labeled nick-translated probe (a 1-kilobase complementary DNA fragment of NMB was radiolabeled using a BRL nick translation kit; the specific activity was approximately 500 cpm/pg). The following day the membrane was washed twice in 1× SSC, 0.1% sodium dodecyl sulfate, at room temperature and twice in 0.1× SSC, 0.1% sodium dodecyl sulfate, at 60°C. The membrane was dried and exposed to Kodak XAR-5 film, with an intensifying screen, at −70°C overnight.

The levels of mRNA transcribed from the genes encoding the NMB and GRP peptides were compared in 15 lung cancer cell lines, using the S1 nuclease protection assay (23). The probes were 170-base single-

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[3] The abbreviations used are: NMB, neuromedin B; GRP, gastrin-releasing peptide; BN, bombesin; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; HPLC, high pressure liquid chromatography; RIA; radioimmunoassay; IC_{50}; 50% inhibitory concentration; SSC, standard saline citrate.

stranded DNA fragments hybridizing to the portion of mRNA encoded in the first exon of the GRP and NMB gene. The ^{32}P-labeled probes (100,000 cpm) were hybridized to equal amounts (10 μg) of total RNA isolated from the lung cancer cell lines. Hybridization reactions were performed in 50 μl of 70% formamide, 400 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA. The samples were heated to 80°C for 2 min and hybridized overnight at 50°C. Samples were diluted to 400 μl with 300 mM NaCl, sodium acetate (pH 4.5), 3 mM $ZnCl_2$, and digested with S1 nuclease (400 units) at 37°C for 2 h. S1-protected probe segments were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel and compared to standards.

Results

NMB Peptides. Lung cancer cell lines were extracted with boiling 2 N acetic acid and assayed for immunoreactive NMB and GRP. Fig. 1 shows that the limit of sensitivity for the NMB RIA was 10 fmol. NMB-32 cross-reacted with the NMB antiserum (94%), whereas GRP did not crossreact (<0.01%). Also, an extract for NCI-H345 yielded a dose-response curve that was parallel to that of NMB or NMB-32. Table 1 shows that immunoreactive NMB (0.1–0.7 pmol/mg of protein) was detected in 69% of the lung cancer cell lines examined. This included 10 of 14 classic SCLC, 2 of 3 variant SCLC, 1 of 3 adenocarcinoma, 1 of 2 large cell carcinoma, 1 of 2 squamous cell carcinoma, 3 of 4 NSCLC neuroendocrine, and 5 of 5 lung carcinoids. In contrast, immunoreactive GRP (0.1–12.9 pmol/mg of protein) was detected in 48% of the lung cancer cell lines examined. This included 11 of 14 classic SCLC, 2 of 4 NSCLC neuroendocrine, and 3 of 5 lung carcinoids. Immunoreactive GRP was not detected in the SCLC variant, adenocarcinoma, large cell carcinoma, and squamous cell carcinoma cell lines. In general, NMB was more widely distributed than was GRP, but the levels of immunoreactive GRP were greater, especially in the classic SCLC cell lines.

NMB was characterized by gel filtration (Fig. 2) and HPLC techniques. Using an extract from NCI-H345, two main peaks of absorbance were detected in fractions 24 (the void volume) and 54 (the included volume). The main peak of immunoreactive NMB eluted in fraction 32 and had a molecular weight similar to that of NMB (M_r 1132). Fig. 3 shows that, using HPLC techniques, the main peak of immunoreactive NMB eluted in fraction 26, as did synthetic NMB, whereas the main peak of immunoreactive GRP eluted in fraction 14, as did synthetic GRP^{18-27}. In contrast, synthetic GRP and NMB-32 eluted in fractions 28 and 32, respectively. These data indicate that SCLC cell line NCI-H345 produces more endogenous GRP^{18-27} than NMB.

NMB mRNA. Fig. 4 shows that a major NMB mRNA species was present at 0.8 kilobase in cell lines NCI-H345 (classic SCLC) and H1581 (adenocarcinoma) but not NCI-H187 (classic SCLC). Due to the relatively low abundance of mRNA, bands were observed using 10 μg of poly(A) RNA but not total RNA.

NMB mRNA was also determined by S1 nuclease protection. Fig. 5 shows that low levels of NMB RNA were present in all 15 cell lines tested, with the exception of NCI-H187 (classic SCLC). In contrast, high levels of GRP mRNA were present in NCI-H1092, H345, and H209 (classic SCLC), as well as NCI-H835 (lung carcinoid), whereas low levels of GRP mRNA were present in NCI-H146 (classic SCLC). GRP mRNA was not detected in cell lines NCI-H1694 and H187 (classic SCLC), H522 and H1581 (adenocarcinoma), N417 (variant SCLC), H1299 (large cell), H727, H720, and UNC-11 (lung carcinoid),

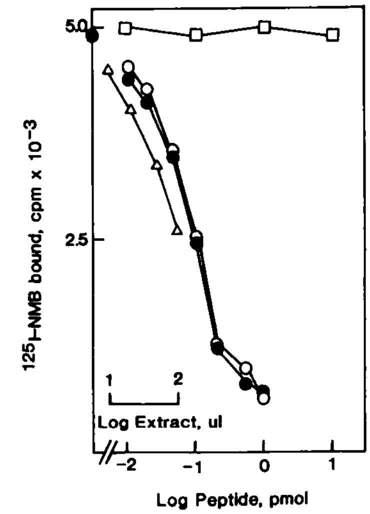

Fig. 1. NMB radioimmunoassay. A dose-response curve for the NMB radioimmunoassay using increasing doses of NMB (●), NMB-32 (○), GRP (□), and NCI-H345 extract (△). The mean value of two determinations is indicated and the SD was 5% of the mean value.

Table 1 *Peptide density in lung cancer cell lines*
The mean values of two determinations are indicated.

Cell line	Peptide density (pmol/mg protein) NMB	BN/GRP
SCLC classic		
NCI-H60	0.2	0.3
NCI-H128	0.1	1.4
NCI-H146	0.1	0.2
NCI-H187	<0.1	<0.1
NCI-H209	0.2	12.9
NCI-H345	0.4	2.6
NCI-H510	0.3	2.4
NCI-N592	0.7	0.3
NCI-H660	<0.1	<0.1
NCI-H1048	0.6	0.5
NCI-H1092	<0.1	3.8
NCI-H1341	0.1	0.1
NCI-H1694	<0.1	<0.1
NCI-H1926	0.1	0.5
SCLC variant		
NCI-H82	0.2	<0.1
NCI-N417	0.1	<0.1
NCI-H446	<0.1	<0.1
Adenocarcinoma		
NCI-H522	<0.1	<0.1
NCI-H1264	<0.1	<0.1
NCI-H1581	0.1	<0.1
Large cell carcinoma		
NCI-H1299	0.1	<0.1
NCI-H1570	<0.1	<0.1
Squamous cell carcinoma		
NCI-H157	0.4	<0.2
NCI-H596	<0.1	<0.1
NSCLC neuroendocrine		
NCI-H460	0.2	0.6
NCI-H810	<0.1	<0.1
NCI-H1155	0.2	<0.1
NCI-H1341	0.1	0.1
Carcinoid		
UNC-11	0.2	<0.1
NCI-H679	0.7	2.7
NCI-H720	0.1	0.1
NCI-H727	0.1	<0.1
NCI-H835	0.7	1.0

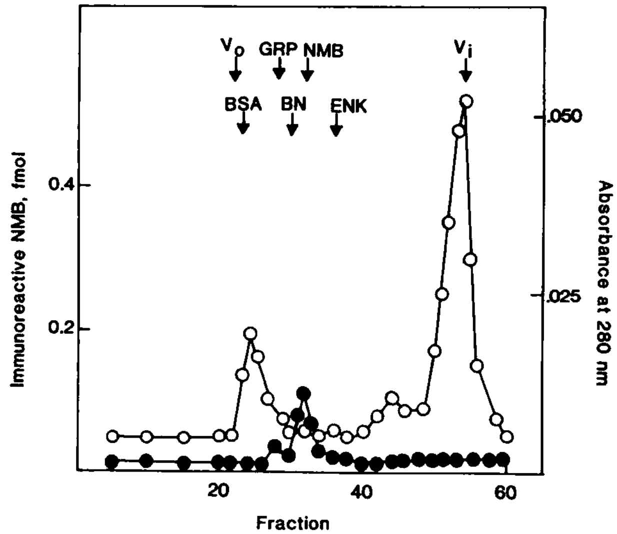

Fig. 2. Gel filtration profile. NCI-H345 extract was loaded on Sephadex G-25 and eluted with 0.1 N acetic acid. The eluted samples were lyophilized and assayed for immunoreactive NMB (●) and absorbance at 280 nm (○). The elution positions of blue dextran (V_o), ^{125}I (V_i), bovine serum albumin (*BSA*), GRP, BN, NMB, and enkephalin (*ENK*) are indicated.

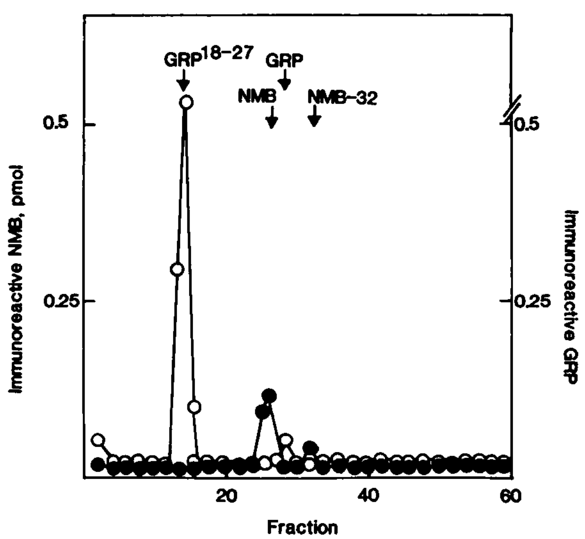

Fig. 3. HPLC profile. NCI-H345 extract was loaded onto a μBondapak C18 column and eluted with 0.25 M triethylammonium phosphate (pH 3.0) and an acetonitrile gradient (18–33%) over a 60-min period; the flow rate was 1 ml/min. The sample was lyophilized and assayed for immunoreactive NMB (●) and GRP (○).

and H460 (NSCLC-neuroendocrine) and the tRNA control. Because the autoradiographic exposure time is equal for samples hybridized to both probes, the exposure time needed to detect the relatively low levels of prepro-NMB mRNA results in overexposure of some of the prepro-GRP mRNA samples. These data indicate that NMB mRNA expression is more widespread than that of GRP but the levels of GRP mRNA are greater in classic SCLC and lung carcinoids.

Discussion

In the mammalian brain, NMB and GRP may function as distinct regulatory peptides. The peptides have different anatomical locations, in that NMB and prepro-NMB mRNA are present in the rat olfactory bulb (24–26), whereas GRP and prepro-GRP mRNA are present in suprachiasmatic nucleus of the hypothalamus (26–28). Also, rat prepro-NMB contains 114 amino acid residues but has a similar sequence as human prepro-NMB (26), whereas rat prepro-GRP contains 142 amino acid residues but has a similar sequence as human prepro-GRP (29). These differences are consistent with the idea that, although these two peptides are structurally similar, their functions might be quite distinct. Previous studies indicate that high levels of GRP and prepro-GRP mRNA are expressed in classic SCLC cells and that GRP functions as an autocrine growth factor in some SCLC cells (4). In the present study, we analyzed NMB expression in a panel of cultured human lung cancer isolates.

Using a heterologous antiserum specific for NMB, immunoreactive NMB was detected in extracts from both SCLC and NSCLC cell lines. Gel filtration and HPLC analysis, indicated that the main peak of immunoreactivity in the SCLC cell line NCI-H345 coincided with NMB, a 10-amino acid peptide, and not NMB-32, a larger precursor form. These results suggest that NCI-H345 possesses the enzymes needed to process NMB from NMB-32 by cleavage with a trypsin-like activity at basic amino acid residues. Similarly, the main peak of GRP immunoreactivity corresponded to GRP$^{18-27}$, which is generated by cleavage of the 27-amino acid GRP in an analogous fashion.

NMB mRNA was detected in cell lines NCI-H209 and H1581 by Northern analysis and S1 nuclease protection. Similarly, each of these cell lines has NMB, by radioimmunoassay. In contrast, cell line NCI-H187 lacked NMB, based on radioimmunoassay, Northern analysis, and S1 nuclease protection data. Also, cell lines NCI-H345, H522, H146, N417, H835, H1299, H727, H460, H720, and H209 were positive for NMB by radioimmunoassay (0.1–0.7 pmol/mg of protein) and S1 nuclease protection. Of particular interest is NCI-N417 (SCLC variant), which lacks GRP but does have GRP receptors and positively responds to NMB in growth assays (30). It is possible that NMB binds to the GRP receptors on this cell line. Also, low levels of NMB mRNA and peptide are present in cell lines NCI-H1694 and H1092, based on radioimmunoassay (0.04 and

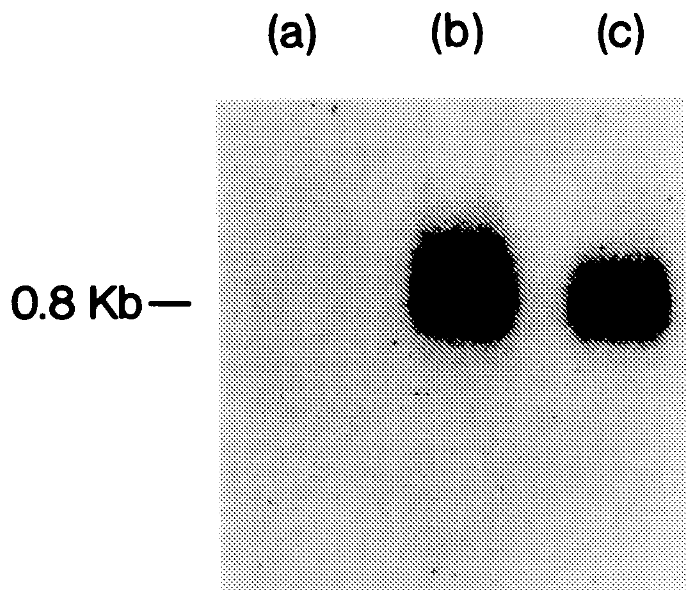

Fig. 4. Northern analysis. RNA was isolated from NCI-H187 (*lane a*), NCI-H1581 (*lane b*), and NCI-H209 (*lane c*), using the GIT procedure, and poly(A) RNA was purified. The sample was then assayed by Northern blot analysis, using a complementary DNA probe for NMB.

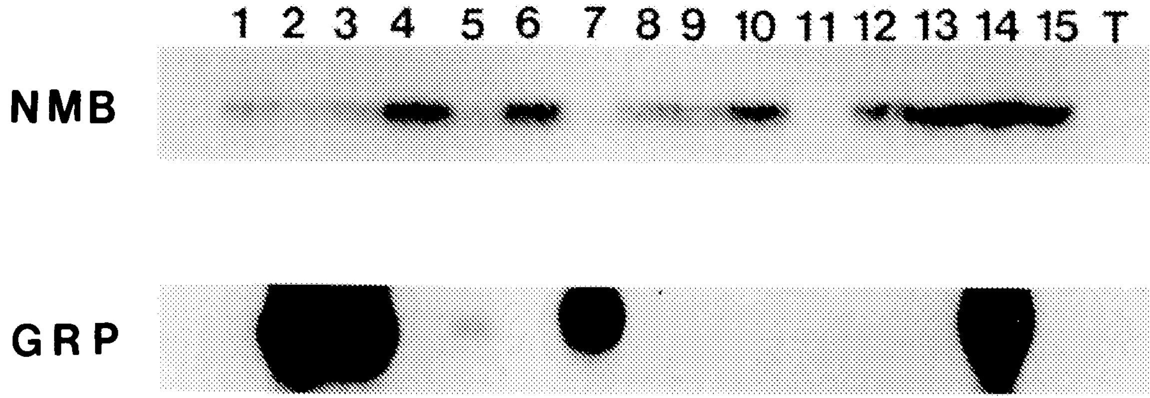

Fig. 5. S1 nuclease protection assay. RNA was obtained from cell lines NCI-H1694 (*lane 1*), NCI-H1092 (*lane 2*), NCI-H345 (*lane 3*), NCI-H522 (*lane 4*), NCI-H146 (*lane 5*), NCI-N417 (*lane 6*), NCI-H835 (*lane 7*), NCI-H1299 (*lane 8*), NCI-H727 (*lane 9*), NCI-H460 (*lane 10*), NCI-H187 (*lane 11*), NCI-H1581 (*lane 12*), NCI-H720 (*lane 13*), NCI-H209 (*lane 14*), and UNC-11 (*lane 15*) and yeast tRNA (*T*) and was analyzed using probes for NMB (*top*) and GRP (*bottom*).

0.02 pmol/mg of protein, respectively) and S1 nuclease protection data.

High levels of GRP mRNA and immunoreactivity were detected in cell lines NCI-H1092, H345, H835, and H209. The levels of immunoreactivity (1.0–12.9 pmol/mg) and mRNA in these cell lines were much higher for GRP than for NMB. Low levels of GRP immunoreactivity and mRNA were present in NCI-H146, whereas they were not detected in cell lines NCI-H1694, H522, N417, H1299, H727, H187, H1581, and UMC-11. It is unknown why cell lines NCI-H720 and H460 had immunoreactive GRP but not GRP mRNA. In general, the distribution of NMB was more widespread than was that of GRP; the levels of GRP were much higher in the positive cell lines. In particular, GRP was present in high concentrations in some classic SCLC, NSCLC neuroendocrine, and lung carcinoid cell lines. In contrast, low levels of NMB were present in SCLC classic, SCLC variant, lung carcinoid, NSCLC-neuroendocrine, adenocarcinoma, large cell carcinoma, and squamous cell carcinoma cell lines. Dense core neurosecretory granules, which possess posttranslational peptide-processing enzymes, are present in SCLC, lung carcinoid, and NSCLC neuroendocrine cell lines (15). The 116-amino acid human prepro-NMB must be first metabolized by a signal protease to yield pro-NMB (92 amino acids) and then metabolized by a trypsin-like enzyme to yield NMB-32GKK and a 57-amino acid carboxyl-terminal extension peptide. Carboxypeptidase-B-like enzymes remove the basic amino acids which flank the NMB sequence, followed by an α-amidation activity that is required to generate the amidated methionine residue at the carboxyl terminus of NMB-32. NMB-32 may then be metabolized by a trypsin-like enzyme to NMB. It remains to be determined if lung carcinoma cells which do not have an abundance of granules, *e.g.,* SCLC variant, adenocarcinoma, large cell carcinoma, and squamous cell carcinoma, have the enzymes needed to metabolize prepro-NMB.

Previously, we determined that immunoreactive GRP was released from SCLC cells by depolarizing stimuli and vasoactive intestinal peptide, which stimulates adenylate cyclase (8, 18). When secreted, GRP may bind to cell surface receptors and function as a regulatory peptide in classic SCLC. It remains to be determined if NMB is secreted from lung cancer cell lines.

Previously, exogenous NMB (10 nM) was demonstrated to increase the clonal growth of three SCLC cell lines 2-fold (30). It is likely that the NMB-dependent growth regulation is mediated through functional receptors in the target SCLC cells. Two pharmacologically distinct bombesin receptors have been described in gastrointestinal tract and central nervous system (31, 32), a GRP-preferring receptor with low affinity for NMB (IC_{50} = 100 nM) and a NMB-preferring receptor with high affinity for NMB (IC_{50} = 1 nM). Molecular cloning studies indicate that both receptors are structurally similar (54% amino acid identity) members of the guanine nucleotide-binding protein-coupled receptor family (32–34). Recently, we found that NMB binds with low affinity (IC_{50} = 200 nM) to GRP receptors on lung cancer cells such as NCI-H720 and with high affinity (IC_{50} = 1 nM) to NMB receptors on NCI-H209.[4] Preliminary studies indicate that both receptors utilize similar signal transduction pathways, including interaction with guanine nucleotide-binding proteins, phosphatidylinositol turnover, and elevation of intracellular calcium.[5] Because NMB stimulates the growth of certain SCLC cell lines, such as NCI-H209, NMB may also function as an autocrine growth factor in lung cancer cells expressing adequate levels of NMB-preferring receptors. It is also possible that NMB-like peptides could stimulate the growth of lung cancer cells expressing GRP-preferring receptors, although much higher levels of the peptide would likely be required. Further studies defining the structure and pharmacology of bombesin receptors in human lung carcinomas will be needed to understand more completely the role of GRP and NMB in stimulating the growth of human lung cancer cells and to assess the utility of specific receptor antagonists to interfere with growth.

In summary, because NMB-like immunoreactivity and NMB mRNA are present in several lung cancer cell lines, NMB may function as a regulatory peptide in lung cancer.

Acknowledgments

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